Effects of *recA* Mutations on Pilus Antigenic Variation and Phase Transitions in *Neisseria gonorrhoeae*

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ABSTRACT

Intragenic recombination between the single complete pilin gene (expression locus) and multiple, distinct, partial pilin gene copies (silent, storage loci) is thought to account for the generation of pilus antigenic diversity and piliation phase (on-off) changes exhibited by *Neisseria gonorrhoeae*. The mechanisms operating in the genomic rearrangements associated with these forms of pilus variation were investigated through the study of isogenic strains of gonococci bearing either wild-type or altered *recA* alleles. Examination of the rates of pilus phase variation and the genetic basis for changes in piliation status displayed by these strains show that *recA* mediated homologous recombination is required for these high frequency events and confirm that the nonpiliated state results from mutations in the expressed pilin gene. In a strain that is deficient in *recA* mediated homologous recombination, pilus phase variation occurs at a 100–1000-fold reduced rate and results predominantly from one class of spontaneous frameshift mutations within the pilin structural gene.

THE presence of pili (or fimbriae) can be correlated with virulence in many gram-negative pathogenic bacteria that inhabit mucosal surfaces of mammals. These proteinaceous appendages have been implicated in bacterial adherence and colonization of the human host by *Neisseria gonorrhoeae*, the causative agent of gonorrhea (SWANSON 1973; PEARCE and BUCHANAN 1978; MCGEE *et al.* 1978). This particular species is remarkable in that intrastrain variants displaying biochemically and antigenically distinct pili arise at readily detectable although undetermined frequencies (VIRJI and HECKELS 1983; HAGBLOM *et al.* 1985; BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1987a, b). The expression of multiple pilin serotypes might enable the microorganism to maintain essential pilus-mediated properties in an immunocompetent host. In addition to this form of heterogeneous pilus expression, piliated (pilus$^+$) gonococci cultivated *in vitro* give rise to nonpiliated and some, but not all, pilus$^-$ variants are capable of spawning pilus$^+$ revertants (SWANSON *et al.* 1985). Such phase transitions between piliated and nonpiliated states are easily recognized by attendant changes in colonial morphology (SWANSON, KRAUS and GOTSCHLICH 1971) and occur with frequencies that are approximately 1,000–10,000-fold higher than the rates of spontaneous mutation.

Biochemical and genetic studies have shown that gonococcal pilins are synthesized as polypeptides approximately 165–170 amino acid (a.a.) residues in length and that their mature polymerized form present in pili lacks the first seven amino terminal residues of the gene's primary translational product (HERMODSON *et al.* 1978; MEYER *et al.* 1984). The pilin molecule displays a conserved amino-terminal domain (a.a. 1–61), a "semivariable" central region in which moderate amino acid substitutions are found (a.a. 62–121 with nonvariable residues at a.a. 79–85, 93–97 and 113–116) and a "hypervariable" segment in which contiguous stretches of residues change (a.a. 135–150 and 159–166) (SCHOOLNIK *et al.* 1984; HAGBLOM *et al.* 1985; BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). The regions that flank the hypervariable region (a.a. 122–134 and 151–158) are conserved and contain cysteine residues thought to be disulfide bonded to one another. The pilin hypervariable domain is the immunodominant portion of purified pili and changes in this region account for some of the demonstrable antigenic differences among gonococcal pili (VIRJI and HECKELS 1983; ROTHBARD, FERNANDEZ and SCHOOLNIK 1984; SWANSON *et al.* 1987a,b). The gonococcal chromosome usually contains a single intact pilin gene (although in one strain, two complete genes have been documented) and multiple copies of variant partial pilin genes that lack transcriptional promoters and amino terminal encoding sequences (MEYER *et al.* 1984; SWANSON *et al.* 1985; HAAS and MEYER 1986; SWANSON *et al.* 1986). It has been shown that pilus antigenic variation and phase transition both correlate with DNA rearrangements within the pilin structural gene and that these events appear to result from genetic exchanges between the complete pilin gene and nonexpressed partial genes (HAGBLOM *et al.* 1985;

BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986; SWANSON *et al.* 1987a,b). In phase changes, pilus$^-$ organisms arise from recombinational processes that generate nonreverting deletion mutations (SWANSON *et al.* 1985) as well as reverting frameshift or missense mutations in the pilin gene (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). Thus, the generation of pilus$^+$ revertants by pilus$^-$ organisms and of pilus antigenic variants by pilus$^+$ organisms result from identical events, that is productive intragenic recombination between the pilin expression locus and incomplete pilin gene copies. By the use of specific oligonucleotide probes, this genetic exchange has been shown to be nonreciprocal in character (SWANSON *et al.* 1986; SWANSON *et al.* 1987a) and resembles what has been termed gene conversion. That term may not be entirely appropriate because gonococci are 1) diplococci whose precise chromosomal interactions and constitution are not presently well understood and 2) extremely competent for transformation when in a piliated state (SPARLING 1966), and since all the products of genetic exchange may not be recoverable as they are following genetic crosses in fungi and yeast.

In order to identify the metabolic reactions and mechanisms that are involved in gonococcal pilin intragenic recombination and to test our current notions about the regulation of pilus expression, we have studied previously described isogenic gonococcal mutants that have defective *recA* alleles with regard to their relative frequency of pilus phase transitions and the genetic events responsible for the phenotypic changes. The results confirm that, in wild type gonococci, alterations in the pilin structural gene account for the nonpiliated state and that these high frequency variations require homologous recombination functions that involve the *recA* gene product. In the absence of functional *recA* gene product, pilus$^-$ variants arise at rates that are at least thirty-fold reduced as compared to the *recA*$^+$ strain and result from one major class of spontaneous frameshift mutations within the pilin gene. The generation of pilus$^+$ revertants by these *recA* pilin mutants occurs at rates that are a 1000-fold lower than that found in wild-type strains and results from true reversions at the same mutational hotspot.

MATERIALS AND METHODS

Bacterial strains: Strains VD300 (pilus$^+$, Δ*pilE2*), VD301 (pilus$^+$, Δ*pilE2*, *recA301*) and VD302 (pilus$^+$, Δ*pilE2*, Δ*recA302*) (KOOMEY and FALKOW 1987) were grown at 37° in 5% CO_2 on clear medium as described before (SWANSON *et al.* 1985). These organisms have a single pilin expression locus designated *pilE1* and have a deletion within the *pilE2* locus that encompasses the complete 4.3-kb *Cla*I fragment previously characterized (MEYER *et al.* 1984). The pilin subunits elaborated by organisms displaying P^{++} colony piliation phenotypes (SWANSON and BARRERA 1983) of each of the strains are biochemically and immunologically indistinguishable from one another. The pilin gene nucleotide sequences and amino acid positions noted here follow the conventions established in prior studies (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). The nucleotide sequence of the VD302 P^{++} pilin gene determined by oligonucleotide primer extension of its pilin mRNA is identical to the DNA sequence of the pilin gene contained in the plasmid pVD203 (BERGSTRÖM *et al.* 1986) with the sole exception of single base difference at nucleotide position 685 that changes an alanine (GCC) at amino acid position 117 to a glycine (GGC). The VD301 P^{++} pilin gene also contains that same alteration as the VD302 pilin gene as well as five other nucleotide changes between nucleotides 591 and 609. Only two of these result in amino acid alterations so that VD301 pilin has lysine rather than glutamine at amino acid position 89 and glutamine rather than lysine at amino acid position 91. Nonpiliated variants derived from these strains were identified by colonial morphology and in cases where their piliation status was in doubt it was confirmed by scanning electron microscopy as described (SWANSON *et al.* 1985). Piliated revertants were assessed by colonial morphology alone.

Phenotypic and genotypic analysis of pilus variants: Expression of pilin protein was examined by immunoblotting of solubilized whole gonococci as detailed (SWANSON *et al.* 1986). The MCO2 monoclonal antibody recognizes an epitope that is present on pilin polypeptides of all of the strains examined to date while the 1H5 monoclonal antibody recognizes an epitope encompassed by amino acid positions 128–141 of pilins expressed by the parental pilus$^+$ variants used here (SWANSON *et al.* 1987b). The preparation of genomic DNA and total RNA as well as the conditions for Southern and Northern blotting were performed as described (SWANSON *et al.* 1985). Pilin mRNAs were sequenced by primer extension using ^{32}P-labeled oligonucleotides. The protocols for this technique and the particular primers used have been published previously (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). For those pilus$^-$ variants that failed to give rise to pilus$^+$ revertants at detectable frequencies on solid media, organisms were cultivated in static broth cultures to selectively enrich the recovery of rare revertants (FROHOLM, JYSSUM and BOVRE 1973). Three-milliliter volumes of tenfold dilutions of these organisms (starting at 10^8 colony-forming units (cfu) per ml) in clear media lacking agar were cultivated without shaking in 12 × 75 mm polystyrene round bottom tubes (Falcon 2054, Becton Dickinson) and incubated at 37° in the presence of 5% CO_2 for at least 36 hr. The pellicle of bacterial growth at the air-liquid interface was carefully subcultured onto solid media and the colonies examined. Variants that failed to show evidence of reversion under these conditions were examined for the loss of the 5′ end of the pilin gene by colony hybridization (GRUNSTEIN and HOGNESS 1975) using the oligonucleotide GCO6 (SWANSON *et al.* 1985) that is complementary to the sequences that encode the first seven amino acids of the pilin polypeptide.

Measurement of the rates of piliation transitions: Rates of piliation transitions were calculated by modification of a published protocol (ENOMOTO and STOCKER, 1975). Single whole colonies of gonococci were excised by stabbing the agar with the broad end of a sterile pasteur pipette and transferred to 1 ml of broth media lacking supplements. After vigorous vortexing and exposure to brief (5–10 sec) pulses of disruption in a sonicating water bath (Astroson model 9, Heat Systems Ultrasonics) to disrupt bacterial aggregates, dilutions were spread so that each plate contained between 50 and 100 well separated colonies. This treatment did not result in decreased viability and in the

case of piliated organisms increased the number of cfu five- to tenfold. The term colony forming unit is used rather than cell since gonococci exist as diplococcal units. After varying time lengths of incubation, at least six colonies of a given variant at a given time point were treated as above and dilutions were plated to calculate viable cfu and to determine the ratio of piliated or nonpiliated colonies (as determined by colonial morphology) to total cfu. Rates of piliation transitions were calculated by the formula $(M/N)/g$ where M/N is the ratio of variants to total cfu and g is the number of generations of growth from a single cfu to the number of cfu within a colony ($\log_2$ of total cfu). The results presented are the averages of these results. All colonies displaying P^{++} or P^{+} morphology contain pilus$^+$ organisms but not all colonies of pilus$^+$ organisms display P^{++} or P^{+} morphotypes (M. KOOMEY, unpublished data). That is to say that some colonies displaying a pilus$^-$ morphology actually consist of pilus$^+$ organisms. Therefore, the rates of pilus$^+$ to pilus$^-$ transitions determined here represent maximal values while the rates of pilus$^-$ to pilus$^+$ transitions represent minimal values.

RESULTS

Effects of altered *recA* alleles on the rates of pilus$^+$ to pilus$^-$ transitions: The *recA* gene from the gonococcal strain MS11$_{mk}$ has been cloned in *Escherichia coli* and derivatives of this cloned gene were used to construct, by transformation-mediated marker rescue, an isogenic set of piliated MS11 derivatives bearing mutated *recA* alleles. The wild-type parent strain, a mutant that displays a reduced proficiency for *recA* associated functions due to an insertion of DNA near the *recA* structural gene (the *recA301* allele) and a mutant that displays an absolute deficiency in *recA* functions due to a deletion that encompasses part of the gene and its flanking sequences (the *ΔrecA302* allele) have been designated strains VD300, VD301 and VD302, respectively (KOOMEY and FALKOW 1987). A modified version of the protocol developed for the study of flagellar phase variation in Salmonella was used to determine the frequency of pilus$^+$ to pilus$^-$ transitions. With strain VD300, it became clear that colonies harvested after 21 hr of growth yielded increased values for the rates of pilus$^+$ to pilus$^-$ transitions when compared to colonies picked before that time (data not shown), a phenomenon that has been previously documented (NORLANDER *et al.* 1979). Since there was little increase in the total cfu in a colony after 21 hr of growth, the increase in proportion of pilus$^-$ variants appears to reflect the selective growth advantage of pilus$^-$ variants generated previous to this period of nonexponential growth. This was not surprising considering that pilus$^+$ organisms form small domed colonies with well defined edges due to their autoagglutinability (KELLOGG *et al.* 1968; SWANSON *et al.* 1971) while pilus$^-$ organisms form flat, spreading colonies and since nonselective subculture on solid media has long been known to result in a loss of piliation and virulence within the gonococcal population (KELLOGG *et al.* 1963). Therefore, in this analysis strains VD300 and VD301 were propagated for 18 hr or less while strain VD302, with a slightly longer generation time, was propagated for 21 hr before harvesting and plating. To minimize the enrichment of pilus$^-$ organisms during the subsequent growth on solid media required to assess piliation, colonial morphology was scored at 17 and 20 hr, respectively. The wild-type strain (VD300) generated pilus$^-$ variants at a rate of 2.2×10^{-5}/cfu/generation while the rate of appearance of such variants in the *recA* insertion mutant (VD301) was reduced to 4.1×10^{-6}/cfu/generation (Table 1). Under these conditions, no pilus$^-$ variants were found among 120,000 colonies examined of the *recA* deletion mutant, strain VD302 ($<6 \times 10^{-7}$/cfu/generation). However, by prolonged growth (42 hr or more) rare pilus$^-$ variants could be recovered from that strain.

TABLE 1

Rates of pilus transitions[a]

	$P^+ \rightarrow P^-$	$P^- \rightarrow P^+$
VD300 (*recA*$^+$)	2.2×10^{-5}	6.5×10^{-4}
VD301 (*recA301*)	4.1×10^{-6}	4.5×10^{-5} (8/18 P$^-$r)
VD302 (*ΔrecA302*)	$<6.0 \times 10^{-7}$	$<5.0 \times 10^{-7}$ (12/12 P$^-$r)

[a] Results are expressed as variants/cfu/generation and are averages with actual values varying ±20%. P$^+$, pilus$^+$; P$^-$, pilus$^-$. Numbers within parentheses indicate the ratio of nonpiliated variants capable of spawning piliated revertants to the total number of nonpiliated variants studied.

Pilus$^-$ variants can be classified into two distinct phenotypes, one is incapable of spawning piliated revertants (P$^-$n) while the other retains this capability (P$^-$r); organisms displaying a P$^-$r phenotype can be further categorized by their synthesis of pilin (P$^-$rp$^+$) or lack thereof (P$^-$rp$^-$). The P$^-$n phenotype results from loss of the single genomic copy of the transcriptional promoter and 5′ end of the pilin gene (SWANSON *et al.* 1985). Organisms of the P$^-$r phenotype have an intact pilin gene and express pilin mRNA that either contains a premature translation termination signal (P$^-$rp$^-$) or encodes full-length, antigenically detectable pilin polypeptide that is not polymerized into pili (P$^-$rp$^+$) (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). In strain MS11$_{mk}$, the P$^-$rp$^+$ phenotype is most often associated with the expression of an "assembly missense" pilin polypeptide bearing amino acid residues 136–148 derived from pilin gene copy 5 of the *pilS1* locus (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986; HAAS and MEYER 1986). The phenotypes and genotypes of independently derived pilus$^-$ variants from strains VD301 and VD302 but not VD300 were investigated since the latter is virtually identical to the MS11$_{mk}$ strain used in previous studies of this kind (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986; KOOMEY and FALKOW 1987).

Characterization of pilus$^-$ variants of the *recA* insertion mutant VD301: Ten of 18 VD301 pilus$^-$

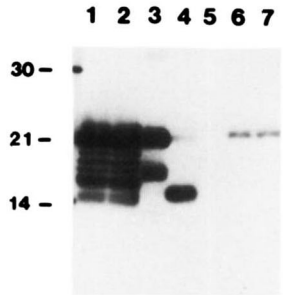

FIGURE 1.—Pilin polypeptides of P^{++} and P^- variants of strains VD301 and VD302 detected by immunoblotting. Whole cell lysates of pilus variants (equivalent to 10^8 cfu) were subjected to SDS-polyacrylamide gel electrophoresis and were immunoblotted with antipilus monoclonal antibody MCO2. Lane 1, VD302 P^{++}; 2, VD301 P^{++}; 3, VD301 P^- #1 (P^-rp^+ phenotype); 4, VD301 P^- #3 (P^-rp^+ phenotype); 5, VD301 P^- #5 (P^-n phenotype); 6, VD302 P^- #1 (P^-rp^- phenotype); 7, VD302 P^- #2 (P^-rp^- phenotype). Numbers on left represent the migration of M_r standards ($\times 10^{-3}$): carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,000.

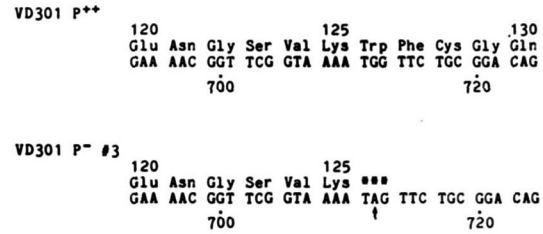

FIGURE 2.—Pilin mRNA sequences of strain VD301 P^{++} and P^- #3 (P^-rp^+ phenotype) variants. Pilin gene nucleotide (underneath) and encoded amino acid positions (above) are according to BERGSTRÖM *et al.* (1986). Sequences start with the codon for amino acid 120 and extend through to the codon for amino acid 130. The arrow denotes the location of a single base change (a G to A transition) within the codon for amino acid 126 of the VD301 P^- #3 transcript that results in translational termination and expression of a truncated pilin polypeptide.

variants displayed P^-n phenotypes as deduced from their failure to revert at detectable frequency, and to express either pilin mRNA or pilin antigen. Southern blotting studies were used to show that the 4.1-kb *Cla*I fragment carrying the pilin gene had undergone a gross rearrangement characteristic of this genotype (SWANSON *et al.* 1985) and confirmed that the genome of these organisms had lost the unique copy of the 5′ end of their pilin gene as seen by their failure to react with an oligonucleotide probe complementary to the region encoding the first seven amino acids of the pilin gene product (data not shown).

The eight remaining $pilus^-$ variants were of the P^-rp^+ class in that they expressed pilin protein detectable by immunoblotting with the broadly cross-reactive monoclonal antibody, MCO2 (SWANSON and BARRERA 1983). The pilins expressed by these $pilus^-$ variants were heterogeneous in electrophoretic mobilities and antigenically distinct from the pilin expressed by their piliated progenitor as seen by their failure to react following immunoblotting with the 1H5 monoclonal antibody, that recognizes a defined linear epitope between amino acids 128–141 (SWANSON *et al.* 1987b) as well as other selected monoclonal antibodies (data not shown). In strain MS11 and its derivatives, P^-rp^+ gonococci elaborate two major species of pilin antigen (M_r = 21,000 and M_r = 16,000–17,000; see Figure 1, lane 3, for example) while $pilus^+$ gonococci produce a single predominant species of the slower migrating (M_r = 21,000) form (SWANSON *et al.* 1985, 1986). Although the biochemical basis for the M_r = 16,000–17,000 class of pilin polypeptide associated with the P^-rp^+ phenotype is not clear, the shared reactivity pattern of both forms with pilin monoclonal antibodies and the facts that these variants have a single active pilin gene and a homogeneous population of pilin mRNA suggest that the lower M_r form is posttranslationally derived from the M_r = 21,000 polypeptide. The properties of seven of the eight $pilus^-$ derivatives expressing pilin in this strain bearing the *recA301* allele were identical to those of analogous variants characterized in $recA^+$ strains and it seems most likely that they arose by recombinational events similar to those previously described. However, in one of the P^-rp^+ variants of this strain (P^-rp^+ #3), a single pilin species of M_r = 15,000 was found (Figure 1, lane 4). The basis for the unique phenotype of this variant became apparent when its pilin mRNA and that of its piliated parent were sequenced by oligonucleotide primer extension and compared (Figure 2). The entire sequences of these two mRNAs were identical except for a single nucleotide difference at the second position of the codon specifying amino acid 126 that creates an amber translational stop codon in the pilin transcript of the $pilus^-$ organism. The relative mobility of the pilin detected by immunoblotting correlated with the truncated 125 amino acid polypeptide predicted by premature translational termination.

Frameshift mutation accounts for phase transition in the absence of homologous recombination functions: Twelve independent $pilus^-$ variants derived from the *recA* deletion mutant (strain VD302) were selected for study. These variants failed to show any evidence of spawning $pilus^+$ revertants despite rigorous examination during numerous daily subcultures nor did they express pilin antigen detectable by standard immunoblotting techniques. They did not, however, correspond to the previously described P^-n phenotype that results from 5′ deletions of the pilin gene since they showed no evidence of gross rearrangement of the pilin expression locus by Southern blotting and they expressed typical levels of full size pilin mRNA as seen by Northern blotting (data not shown). In light of this data, immunoblotting was repeated using increased amounts of whole cell lysates and the MCO2 monoclonal antibodies (Figure 1). Under these conditions, a faint reactive band of M_r = 21,000 was detected in these samples but not in iden-

tically prepared lysates of VD301 P⁻n variants with the nonreverting pilin gene deletion mutations. The pilin antigen found in the VD302 pilus⁻ variants represented either an extremely low level of expression by all organisms (a leaky phenotype) or pilin synthesised by pilus⁺ revertants too rare to be detected by our standard visual assay. Piliated strains of *Neisseria meningitidis* and other gram-negative species form a pellicle when cultured in static broth media and such conditions can be used to selectively enrich for pilus⁺ organisms and facilitate their recovery (FROHOLM, JYSSUM and BOVRE 1973). When grown in this manner, all twelve VD302 pilus⁻ variants formed surface pellicles, which when subcultured onto solid media, regularly yielded pilus⁺ revertants while identical cultures of VD300 and VD301 variants with pilin gene deletion mutations failed to do so. The pilus⁻ variants of the *recA* deletion mutant therefore displayed a P⁻rp⁻ phenotype.

Under the immunoblotting conditions used above it was also noted that samples of piliated organisms demonstrated multiple discrete reactive species between M_r = 21,000 and 14,000 that were absent in samples derived from organisms of the P⁻rp⁺ phenotype and that the patterns of VD301 P⁺⁺ and VD302 P⁺⁺ lysates were indistinguishable (Figure 1). The correlation of the expression of these minor pilin forms of altered mobility and piliation has been confirmed repeatedly by analysis of these and other strains (M. KOOMEY, unpublished data).

The pilin mRNAs expressed by the pilus⁺ progenitor of strain VD302, three pilus⁻ variants and a pilus⁺ revertant of each of these were sequenced by oligonucleotide primer extension. The sequences obtained from the nonpiliated variants were identical to one another but differed from that derived from the piliated predecessor and revertants by the deletion of a single nucleotide (Figure 3). Whereas the pilin transcripts from the pilus⁺ organisms contained eight cytidines at nucleotide positions 559 through 566 (encoding amino acids 75–77), the pilus⁻ transcripts displayed a deletion of one of these cytidines. The loss of any one of these eight cytidines results in a frameshift mutation, premature translational termination and expression of an immunologically undetectable, truncated polypeptide. Reinsertion of a cytidine within this same stretch of nucleotides restored the integrity of the reading frame and therefore, the pilus⁺ revertants resulted from back mutations owing to true reversions.

Effects of altered *recA* alleles on the rates of productive pilin gene recombination: With the exception of the *recA* deletion mutant noted, the spawning of pilus⁺ revertants by pilus⁻ organisms and the direct generation of variants expressing antigenically distinct pili by pilus⁺ organisms both correlate with

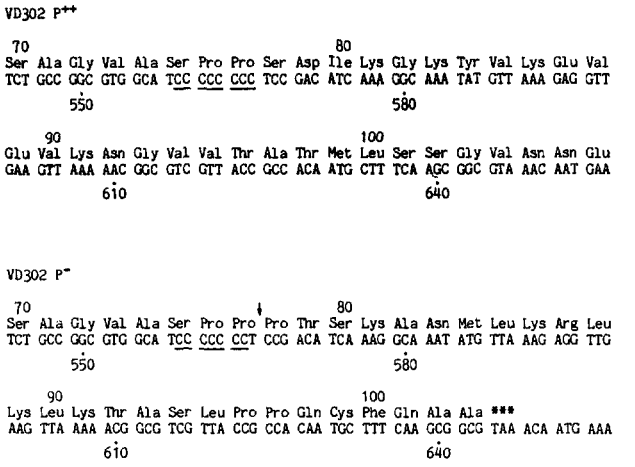

FIGURE 3.—Location and determination of the frameshift mutation of the pilin structural gene associated with phase variation in strain VD302. Pilin gene nucleotide (underneath) and encoded amino acid positions (above) are according to BERGSTRÖM *et al.* (1986). Sequences start with the codon for amino acid 70 and extend through to the codon for amino acid 107. On the top is shown the mRNA sequence determined for the VD302 P⁺⁺ progenitor and piliated revertants derived from independent VD302 P⁻ variants. On the bottom is shown the mRNA sequences found in three independent VD302 P⁻ variants. The arrow denotes the frameshift in translational reading frame that results from the deletion of any one of eight cytidines between nucleotides 559 and 566.

gene conversion that results in the synthesis and expression of a novel functional pilin protein. Since the elaboration of pili of altered antigenicity is not always associated with a discernible change in colonial morphology, the frequency of productive pilin gene conversion can most readily be assessed as the frequency with which reverting pilus⁻ organisms give rise to pilus⁺ organisms. These data were determined for two independent variants displaying the P⁻rp⁺ phenotype derived from both strain VD300 and VD301 (Table 1). The values found using the *recA*⁺ strain (VD300) were very similar to that previously reported for strain $MS11_{mk}$ (SWANSON *et al.* 1985) while those obtained from the *recA* insertion mutant (VD301) were about tenfold lower than that seen with the wild-type strains. These frequencies were much higher than that found for the reverting pilus⁻ variants derived from the *recA* deletion mutant, strain VD302 ($<5 \times 10^{-7}$/cfu/generation).

DISCUSSION

Past studies and the work detailed here have shown that nonpiliated variants of gonococci arise in vitro as a result of mutations within the single complete pilin structural gene. The reduced rates of phase variation and the phenotypes displayed by strain VD301 that expresses a reduced proficiency in *recA* associated functions strongly suggests that *recA* gene product is involved in pilin gene conversion events. The approximate tenfold reduction in the rates of pilus⁻ to pilus⁺

transitions found for this strain parallels similar decreases in two other *recA* associated functions, transformability with chromosomal markers and UV resistance, exhibited by this strain (KOOMEY and FALKOW 1987). Two classes of pilin gene lesions are found in recombination proficient derivatives of strain MS11: 1) nonreverting deletion mutations and 2) reverting frameshift, nonsense or missense mutations. The loss of the unique 5′ end of the pilin gene that occurs in nonreverting pilus$^-$ variants can result from either a conventional deletion of those sequences whose endpoints lie within the 3′ end of the expression site and the tandemly arrayed partial pilin gene copies that are found immediately upstream (SEGAL *et al.* 1985; SWANSON and KOOMEY 1985; SWANSON *et al.* 1986) or from the eviction of these sequences by a gene conversion event (SWANSON *et al.* 1985). The failure to detect such nonreverting deletion mutants in the strain carrying the Δ*recA302* allele (VD302) supports the contention that *recA*$^+$ function is required for, or at least significantly facilitates, some part of the deletion process (ALBERTINI *et al.* 1982). Previous investigations of pilin frameshift and missense mutations found in reverting pilus$^-$ variants of strain MS11$_{mk}$ (responsible for the P$^-$rp$^-$ and P$^-$rp$^+$ phenotypes, respectively) have demonstrated that these forward mutations encompass clustered multiple nucleotide substitutions as do the back mutations present in pilus$^+$ revertants (BERGSTRÖM *et al.* 1986; SWANSON *et al.* 1986). Prior to those observations, such processes of reversible phenotypic variation involving mutation mediated by gene conversion had not been documented in prokaryotes. The finding of one pilus$^-$ variant in strain VD301, of the eighteen examined, that contains a single base change shows that such mutations do occur in recombination proficient strains although their presence is obscured by the more prevalent variants bearing multiple substitutions. It also shows that the P$^-$rp$^+$ phenotype can result from pilin gene nonsense mutations as well as from assembly missense mutations.

Data obtained using the VD302 strain that bears the Δ*recA302* allele and is deficient in *recA* mediated functions provide substantial evidence that homologous recombination functions are required for pilin gene conversion associated with phase variation and the generation of antigenically diverse pilins. Phase variation occurs at greatly reduced rates in this strain by reversible spontaneous frameshift mutation within a stretch of redundant C/G basepairs. This region represents a classical example for such alterations (STREISINGER and OWEN 1985) and virtually identical mutational hotspots have been documented in prokaryotes (OESCHGAR and HARTMAN 1970; CALOS and MILLER 1981) and eukaryotes (WILSON *et al.* 1986). The deletion (as opposed to the insertion) of a C/G pair in the three pilus$^-$ variants we studied supports the contention that base removal predominates in spontaneous frameshift mutations of such sequences in bacteria (ROTH 1974; STREISINGER and OWEN 1985). Another frameshift mutation in this same stretch of monotonous nucleotides was previously found in a pilus$^-$ variant of strain MS11$_{mk}$ but in that case the forward mutation resulted from the insertion of a cytidine resulting in nine cytidines (BERGSTRÖM *et al.* 1986). The contention that this latter mutation arose de novo from a recombinational event is supported by the data found here. These sequences reside in what is known to be a variable segment of the gene (BERGSTRÖM *et al.* 1986); so one would expect that the introduction of the Δ*recA302* allele into variants with differing sequences in that region might reveal other hotspots for pilin gene mutation. Although all of the pilus$^-$ variants examined here and in our previous studies carry pilin mutations, a mutation in accessory genes involved in pilin expression, transport or assembly would also display a pilus$^-$ phenotype. The introduction of the *recA* deletion mutation into the MS11 variant that has two complete pilin genes would appear to be a straight forward way to facilitate the search for such mutants and the identification of those genes and gene products. In that case, pilus$^-$ variants with second site mutations that preclude the biogenesis of pili, but not pilin synthesis, or that coordinately repress pilin gene expression should predominate.

The precise role played by the *recA* gene product in catalyzing the DNA rearrangements of pilin gene conversion is obscured by the pleiotropic nature of *recA* mutations (CLARK 1973) and although it is necessary for the process, it alone is probably not sufficient. It should be noted in this regard that the mutations in both strains carrying altered *recA* alleles involve uncharacterized flanking DNA sequences that may contain other genes that effect pilin gene recombination, as previously acknowledged (KOOMEY and FALKOW 1987) and experiments to address this possibility in a definitive manner will require the construction of mutants bearing lesions wholely contained within the *recA* structural gene. Since gonococci are known to be highly competent for transformation using chromosomal DNA, they clearly have a well developed system for the mismatch repair of DNA heteroduplexes. It seems likely then that pilin gene conversion occurs by similar mismatch repair of heteroduplexes formed at the pilin expression locus between resident sequences and those that originate from the multiple partial pilin genes. The *recA* gene product would probably be involved in both the initial pairing and heteroduplex formation as well as in the repair that follows. Of more pertinence to the study of microbial pathogenesis, this work implies that the

recA gene plays an indirect role in the virulence of *N. gonorrhoeae*. Since it has previously been shown to be involved in amplification of toxin genes of *Vibrio cholerae* (GOLDBERG and MEKALANOS 1986) and in the instability of capsule production in *Haemophilus influenzae* type b (HOISETH, MOXON and SILVER 1986) it appears that homologous recombination mediated in part by the *recA* gene product may be a fundamental mechanism by which pathogenic bacteria modulate the expression of virulence associated genes that exist as duplicated elements within the chromosome.

Genomic rearrangement processes can be usefully categorized into two groups, those which are programmed and those which are unprogrammed (SIMON and HERSKOWITZ 1985). Programmed rearrangements are by their nature stereotypic with the molecular events responsible for variable gene expression being predictable while unprogrammed rearrangements represent more stochastic processes in which the genetic basis underlying the expression of a particular phenotype cannot be inherently deduced. Flagellar phase variation in Salmonella and phase variation of type 1 pili in *E. coli* both involve inversion of DNA segments with transcriptional promoters mediated by *recA* independent site specific recombination and therefore are programmed rearrangements (ZIEG, HILMEN and SIMON 1978; SILVERMAN and SIMON 1980; ABRAHAM *et al.* 1985). In this context, most pilus$^+$ to pilus$^-$ transitions in *N. gonorrhoeae* are unprogrammed genetic rearrangements that arise by what might most appropriately be termed abortive gene conversion while pilus$^-$ to pilus$^+$ transitions and pilus antigenic variation processes are programmed events and result from productive gene conversion. That these gene conversion events may be "error prone" as evidenced in some pilus transitions correlates with the previously established association between recombination and mutation (EISENSTARK 1977; WALKER 1984). Not surprisingly, the effect that different *recA* alleles have on the rates of pilin mutation appears to be unique to that particular gene since the mutation frequencies of other genetic markers are not significantly altered in these strains.

The biological significance of nonpiliated gonococci is obscured by the facts that they clearly represent pilin mutants which arise under the selective pressure of laboratory culture conditions and appear at rates significantly lower than the rate of productive intragenic recombination. Although pilus phase transitions may not be directly pertinent to the pathogenesis of gonococcal infection, their study has provided valuable information concerning the mechanisms of pilus antigenic variation and yielded insights which may be relevant to related prokaryotic and eukaryotic systems which are currently less amenable to genetic manipulation and analysis.

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LITERATURE CITED

ABRAHAM, J. M., C. S. FREITAG, J. R. CLEMENTS and B. I. EISENSTEIN, 1985 An invertible element of DNA controls phase variation of type 1 fimbriae of *Escherichia coli*. Proc. Natl. Acad. Sci. USA *82:* 5724–5727.

ALBERTINI, A. M., M. HOFER, M. P. CALOS and J. H. MILLER, 1982 On the formation of spontaneous deletions: the importance of short sequence homologies in the generation of large deletions. Cell **29:** 319–328.

BERGSTRÖM, S., K. ROBBINS, J. M. KOOMEY and J. SWANSON, 1986 Piliation control mechanisms in *Neisseria gonorrhoeae*. Proc. Natl. Acad. Sci. USA **83:** 3890–3894.

CALOS, M. P., and J. H. MILLER, 1981 Genetic and sequence analysis of frameshift mutations induced by ICR-191. J. Mol. Biol. **153:** 39–66.

CLARK, A. J., 1973 Recombination-deficient mutants of *E. coli* and other bacteria. Annu. Rev. Genet. **7:** 67–86.

EISENSTARK, A., 1977 Genetic recombination in bacteria. Annu. Rev. Genet. **9:** 369–396.

ENOMOTO, M., and B. A. D. STOCKER, 1975 Integration, at *Hag* or elsewhere, of *H2* (phase 2 flagellin) genes transduced from *Salmonella* to *Escherichia coli*. Genetics **81:** 595–614.

FROHOLM, L. O., K. JYSSUM and K. BOVRE, 1973 Electron microscopical and cultural features of *Neisseria meningitidis* competence variants. Acta Pathol. Microbiol. Scand. Sec. B **81:** 525–537.

GOLDBERG, I., and J. J. MEKALANOS, 1986 Effect of a *recA* mutation on cholerae toxin gene amplification and deletion events. J. Bacteriol. **165:** 723–731.

GRUNSTEIN, M., and D. S. HOGNESS, 1975 Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:** 3961–3965.

HAAS, R., and T. F. MEYER, 1986 The repertoire of silent pilus genes in *Neisseria gonorrhoeae*: evidence for gene conversion. Cell **44:** 107–115.

HAGBLOM, P., E. SEGAL, E. BILLYARD and M. SO, 1985 Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature **315:** 156–158.

HERMODSON, M. A., K. C. S. CHEN and T. M. BUCHANAN, 1978 Neisseria pili proteins: amino-terminal amino acid sequences and identification of an unusual amino acid. Biochemistry **17:** 442–445.

HOISETH, S. K., E. R. MOXON and R. P. SILVER, 1986 Genes involved in *Haemophilus influenzae* type b capsule expression are part of an 18-kilobase tandem duplication. Proc. Natl. Acad. Sci. USA **83:** 1106–1110.

KELLOGG, D. S., W. L. PEACOCK, W. E. DEACON, L. BROWN and C. I. PIRKLE, 1963 *Neisseria gonorrhoeae*. I. Virulence genetically linked to colonial variation. J. Bacteriol. **85:** 1274–1279.

KELLOGG, D. S., I. R. COHEN, L. C. NORINS, A. L. SCHROETER and G. REISING, 1968 *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96:** 596–605.

KOOMEY, J. M., and S. FALKOW, 1987 Cloning of the *recA* gene of *Neisseria gonorrhoeae* and construction of gonococcal *recA* mutants. J. Bacteriol. **169:** 790–795.

MCGEE, Z. A., M. A. MELLY, C. R. GREGG, R. G. HORN, D. TAYLOR-ROBINSON, A. P. JOHNSON and J. A. MCCUTCHAN, 1978 Virulence factors of gonococci: studies using human fallopian tube organ cultures. pp 258–262. In: *Immunology of Neisseria gonorrhoeae*, Edited by G. F. Brooks, E. C. Gotschlich,

K. N. Holmes and W. D. SAWYER. American Society of Microbiology, Washington, D.C.

MEYER, T. F., E. BILLYARD, R. HAAS, S. STORZBACH and M. SO, 1984 Pilus genes of *Neisseria gonorrhoeae*: chromosomal organization and DNA sequence. Proc. Natl. Acad. Sci. USA **81:** 6110–6114.

NORLANDER, L., J. DAVIES, A. NORQVIST and S. NORMARK, 1979 Genetic basis for colonial variation in *Neisseria gonorrhoeae*. J. Bacteriol. **138:** 762–769.

OESCHGAR, N., and P. E. HARTMAN, 1970 ICR-induced frameshift mutations in the histidine operon of *Salmonella*. J. Bacteriol. **101:** 490–504.

PEARCE, W. A., and T. M. BUCHANAN, 1978 Attachment role of gonococcal pili. Optimum conditions and quantitation of adherence of isolated pili to human cells in vitro. J. Clin. Invest. **61:** 931–943.

ROTH, J. R., 1974 Frameshift mutations. Annu. Rev. Genet. **8:** 319–346.

ROTHBARD, J. B., R. FERNANDEZ and G. K. SCHOOLNIK, 1984 Strain specific and common epitopes of gonococcal pili. J. Exp. Med. **160:** 208–221.

SCHOOLNIK, G. K., R. FERNANDEZ, J. Y. TAI, J. ROTHBARD and E. C. GOTSCHLICH, 1984 Gonococcal pili: primary structure and receptor binding domain. J. Exp. Med. **159:** 1351–1370.

SEGAL, E., E. BILLYARD, M. SO, S. STORZBACH and T. F. MEYER, 1985 Role of chromosomal rearrangement in *N. gonorrhoeae* pilus phase variation. Cell **40:** 293–300.

SILVERMAN, M., and M. SIMON, 1980 Phase variation: genetic analysis of switching mutants. Cell **19:** 845–854.

SIMON, M., and I. HERSKOWITZ, 1985 Introduction. pp xv–xix. In: *Genomic Rearrangements. UCLA Symposia on Molecular Biology, New Series*, Edited by M. SIMON and I. HERSKOWITZ. Alan R. Liss, New York.

SPARLING, P. F., 1966 Genetic transformation of *Neisseria gonorrhoeae* to streptomycin resistance. J. Bacteriol. **92:** 1364–1371.

STREISINGER, G., and J. E. OWEN, 1985 Mechanisms of spontaneous and induced frameshift mutation in bacteriophage T4. Genetics **109:** 633–659.

SWANSON, J., 1973 Studies on gonococcus infection. IV. Pili: their role in attachment of gonococci to tissue culture cells. J. Exp. Med. **137:** 571–589.

SWANSON, J., and O. BARRERA, 1983 Gonococcal pilus subunit size heterogeneity correlates with transitions in colony piliation phenotype, not with changes in colony opacity. J. Exp. Med. **158:** 1459–1472.

SWANSON, J., and J. M. KOOMEY, 1985 Do changes in pilus expression for *Neisseria gonorrhoeae* involve chromosomal rearrangement? pp 137–151. In: *Genome Rearrangements. UCLA Symposia on Molecular Biology, New Series*, Edited by I. HERSKOWITZ and M. SIMON. Alan R. Liss, New York.

SWANSON, J., S. J. KRAUS and E. C. GOTSCHLICH, 1971 Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. **134:** 886–906.

SWANSON, J., S. BERGSTRÖM, O. BARRERA, K. ROBBINS and D. CORWIN, 1985 Pilus-gonococcal variants: evidence for multiple forms of piliation control. J. Exp. Med. **162:** 729–744.

SWANSON, J., S. BERGSTRÖM, K. ROBBINS, O. BARRERA, D. CORWIN and J. M. KOOMEY, 1986 Gene conversion involving the pilin structural gene correlates with pilus$^+$ ⇆ pilus$^-$ changes in *Neisseria gonorrhoeae*. Cell **47:** 267–276.

SWANSON, J., K. ROBBINS, O. BARRERA, D. CORWIN, J. BOSLEGO, J. CIAK, M. BLAKE and J. M. KOOMEY, 1987a Gonococcal pilin variants in experimental gonorrhea. J. Exp. Med. **165:** 1344–1357.

SWANSON, J., K. ROBBINS, O. BARRERA and J. M. KOOMEY, 1987b Gene conversion variations generate structurally different pilin polypeptides in *Neisseria gonorrhoeae*. J. Exp. Med. **165:** 1016–1025.

VIRJI, M., AND J. E. HECKELS, 1983 Antigenic cross-reactivity of *Neisseria* pili: investigation with type- and species-specific monoclonal antibodies. J. Gen. Microbiol. **129:** 2761–2768.

WALKER, G. C., 1984 Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48:** 60–93.

WILSON, J. B., A. HAYDAY, S. COURTNEIGE and M. FRIED, 1986 A frameshift at a mutational hotspot in the polyoma virus early region generates two new proteins that define T-antigen functional domains. Cell **44:** 477–487.

ZIEG, J., M. HILMEN and M. SIMON, 1978 Regulation of gene expression by site-specific inversion. Cell **15:** 237–244.

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